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Analytical validation of a standardised scoring protocol for Ki67 immunohistochemistry on breast cancer excision whole sections: an international multicentre collaboration

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45 *Aims:* The nuclear proliferation marker Ki67 assayed by immunohistochemistry has multiple
 46 potential uses in breast cancer, but an unacceptable level of interlaboratory variability has

hampered its clinical utility. The International Ki67 in Breast Cancer Working Group has undertaken a systematic programme to determine whether Ki67 measurement can be analytically validated and standardised among laboratories. This study addresses whether acceptable scoring reproducibility can be achieved on excision whole sections.

Methods and results: Adjacent sections from 30 primary ER⁺ breast cancers were centrally stained for Ki67 and sections were circulated among 23 pathologists in 12 countries. All pathologists scored Ki67 by two methods: (a) global: four fields of 100 tumour cells each were selected to reflect observed heterogeneity in nuclear staining; (b) hot-spot: the field with highest apparent Ki67 index was selected and up to 500 cells scored. The intraclass correlation coefficient (ICC) for the global method [confidence interval (CI) = 0.87; 95% CI = 0.799–0.93] marginally met the prespecified success criterion (lower 95% CI \geq 0.8), while the ICC for the hot-spot method (0.83; 95% CI = 0.74–0.90) did not. Visually, interobserver concordance in location of selected hot-spots varies between cases. The median times for scoring were 9 and 6 min for global and hot-spot methods, respectively.

Conclusions: The global scoring method demonstrates adequate reproducibility to warrant next steps towards evaluation for technical and clinical validity in appropriate cohorts of cases. The time taken for scoring by either method is practical using counting software we are making publicly available. Establishment of external quality assessment schemes is likely to improve the reproducibility between laboratories further.

Introduction

The nuclear antigen recognised by the Ki67 antibody is expressed in proliferating cells but absent in resting cells.¹ Since its discovery in 1983 by Gerdes *et al.*,¹ Ki67 assessed by immunostaining

has been studied extensively as a prognostic^{2–11} and predictive^{4,6,9,12,13} marker, predominantly in hormone receptor-positive breast cancer, but also in other tumours.^{14–18} For example, presurgical Ki67 has been shown to be a marker for recurrence-free survival¹⁹ and, in the neoadjuvant setting, a marker for endocrine-resistant tumour that may require more aggressive treatment.²⁰ Excellent intra-observer reproducibility under controlled pre-analytical and staining conditions²¹ has contributed to the body of evidence showing the potential of Ki67 immunohistochemistry assay to be implemented in hospital laboratories as a cost-effective part of clinical management.^{22–24} However, poor interobserver reproducibility and variability due to technical aspects of the assay has limited its adoption in clinical practice.^{4,9,25–28}

The International Ki67 Working Group (IKWG) has undertaken a systematic multiphase programme to determine whether Ki67 scoring can be standardised and analytically validated throughout laboratories.^{9,21,29,30} In Phase I, as assessed by the intraclass correlation coefficient (ICC) estimate of interobserver reproducibility, differences in pathologists' visual interpretation were the main source of variability (ICC = 0.71, 95% credible interval (CI) = 0.47–0.78).²¹ Greater concordance was achieved in Phase II, at least on tissue microarrays, when pathologists were trained to calibrate and standardise scoring according to a clearly defined methodology (ICC = 0.94, 95% CI = 0.90–0.97).²⁹ However, in clinical practice, decisions are made on core-cut biopsy or excision specimens, which require general assessment of the entire sample and selection of areas for formal counting. Therefore, in Phase IIIA, we assessed whether acceptable performance could be achieved on core-cut biopsies using a standardised method with two distinct methods of scoring field selection: global (four representative fields, counting 100 nuclei each) and hot-spot (one field with highest Ki67, counting 500 nuclei). The global method achieved

acceptable interobserver reproducibility (ICC = 0.87; 95% CI = 0.81–0.93) according to our prespecified criteria, whereas the hot-spot method did not (ICC = 0.84; CI = 0.77–0.92).³⁰

The current study represents the final Phase (IIIB) of the visual scoring analytical validity programme, wherein we assess whether acceptable performance can be achieved on centrally stained excision whole sections using the scoring method established on core-cut biopsies. Future studies will be required to evaluate variability due to staining and pre-analytical aspects of the assay.

Materials and methods

This study was approved by the British Columbia Cancer Agency Clinical Research Ethics Board (H10-03420). All specimens used in this study were donated by patients who signed institutionally appropriate consent forms, were excess to diagnostic requirements, and ethically available for quality control studies.

CASE SELECTION AND SAMPLE PREPARATION

Excision blocks from 30 oestrogen receptor (ER)-positive breast cancer cases were selected: 15 from the Phase IIIA study³⁰ and 15 from Kawasaki Medical School Hospital, Kurashiki, Japan (Supporting information, Figure S1). Case selection was irrespective of patients' age at diagnosis, tumour grade, size or nodal status. The clinicopathological characteristics of these 30 cases are shown in Supporting information, Table S1. All blocks were sectioned and stained in the Royal Marsden Hospital Histopathology Department using monoclonal antibody MIB1 at dilution 1:50 (Dako UK, Ely, UK) using an automated staining system (Ventana Medical Systems, Tucson, AZ, USA) according to criteria established by the IKWG.⁹ Sections from the same block were stained

in a single immunohistochemistry run, except for four cases where the staining was performed in two different runs. This approach effectively controls for any technical variation in staining.

SAMPLE DISTRIBUTION

Twenty-four volunteer pathologists representing 24 institutions from 12 countries, most of whom participated in the Phase IIIA study, were invited to participate.

Six adjacent sections from each of the 30 excision blocks were centrally stained: the first with haematoxylin and eosin (H&E), the second with p63 (myoepithelial marker, to assist the identification of invasive foci) and the third to sixth with Ki67 (designated as slide sets 1–4). To facilitate application to the general histopathology laboratory environment, physical glass slides (as opposed to virtual slide images) were distributed to the volunteer pathologists. Because the accumulated delays required would have made the study impractical if all pathologists reviewed the same physical glass slides, participating pathologists were divided into four groups and were given one of the four sets of Ki67 slides to score. The H&E and p63 reference slides were made available online as digital images. Twenty-three pathologists successfully completed the study.

SCORING PROTOCOL

All pathologists were specifically trained to score Ki67 with emphasis on having a very low threshold for appreciating ‘brown stain’ and the principles of standardised regions for nuclei counting, through the publicly available proficiency training module (<http://www.gpec.ubc.ca/calibrator>) that was initially used in the Phase II study.²⁹ The detailed scoring protocol is found in the Supporting information document:

‘ki67p3b_scoring_protocol.pdf’. A modified version of the scoring software used in this study is available freely from the Google Play and Apple iTunes store (search term: ‘Ki67’).

SCORING METHODS

The scoring methods used were the same as those employed in the Phase IIIA study:³⁰ (1) a global assessment that is weighted according to the estimated percentage of the total cancer area covered by each of high, medium, low or negligible Ki67 staining levels; (2) an unweighted global assessment; and (3) assessment of Ki67 only in a ‘hot-spot’ area.

Global methods attempt to derive an average score across all the tissue available for assessment. In the weighted and unweighted global methods, Ki67 index counting was performed in the same fashion, but the final Ki67 score was derived differently. Adapted from a scoring protocol that has been used routinely in the Dowsett laboratory,³¹ these two global methods require the pathologist to first assess staining heterogeneity by estimating the percentages of the invasive tumour component of the slide exhibiting relatively high, medium, low or negligible Ki67 staining frequencies. Based on these estimates, an algorithm (Supporting information, Figure S2) dictates the required number of fields to select and score for each Ki67 staining frequency (irrespective of staining intensity, totalling up to four fields). This algorithm was designed such that the four (or fewer) selected scoring fields would capture the full range of staining frequencies, while at the same time be reflective of the proportion in staining frequencies heterogeneity. Up to 100 invasive tumour nuclei within each field are counted using a ‘typewriter’ pattern (Supporting information, Figure S3), similar to how a tissue microarray core was scored in the Phase II study.²⁹

The hot-spot method requires the pathologist to visually select one high-power field with the highest apparent staining rate and, within that area only, count up to 500 invasive tumour nuclei in a ‘typewriter’ pattern.

STATISTICAL ANALYSES

Prespecified criterion for success

Prior to data collection it was hypothesised that at least one of the scoring methods would have an associated ICC statistically greater than 0.80 (ICC of 0.8 being considered as good concordance³²). For planning purposes, power calculations performed under a variety of scenarios considered to represent good reproducibility (and similar to the results observed in the Phase II study) showed that with at least 21 participating pathologists scoring 30 cases, there would be 80% power to exclude ICCs lower than the pre-specified ICC of 0.8 from a 95% credible interval for a given scoring method.

Ki67 score

The Ki67 score was defined as in the Phase IIIA study.³⁰ Positive staining was defined as any brown stain in the nucleus above background, with reference available as needed to provide standard sample images; negative staining was scored when an invasive cancer cell showed only a blue counterstained nucleus. The unweighted global and hot-spot scores were simply the total number of positively stained tumour nuclei counted divided by the total number of tumour nuclei counted. The weighted global score was derived with tumour nuclei counts in each assessed field weighted by the estimated percentage of the total cancer area covered by each of high, medium, low or negligible Ki67 staining levels. As in our previous studies, to satisfy model assumptions of

normality and constant variance, for statistical analyses the Ki67 score is converted to a logarithmic scale by adding 0.1% and applying a log base 2 transformation.

ICC estimates (ranging from 0 to 1, with 1 representing perfect reproducibility) were computed as previously reported in the Phase IIIA study.³⁰ Briefly, variance component analyses were performed to quantify the contributions from the following sources of variability: scoring pathologist (observer), patient tumour (biological variation – each excision block represents a unique patient) and section of the excision block. Similar to the Phase IIIA study, same-section and different-section ICCs were computed. Same-section refers to pathologists scoring the same excision whole section physical slides, while different-section refers to pathologists scoring different physical slides that represent serial sections cut from the same original excision blocks. Credible intervals for the variance components and the ICCs were obtained using the Markov Chain Monte Carlo routines for fitting generalised linear mixed models.

All data analyses were performed using R version 3.3.2.³³ Sources of variation in log2-transformed Ki67 scores were analysed using random effects models as implemented in the R packages lme4 and MCMCglmm. Data were visualised using heat maps, box-plots and spaghetti plots.

Results

ICC OF Ki67 ACCORDING TO SCORING METHOD

The different-section ICC estimate for the weighted global scores was 0.87 (95% CI = 0.799–0.93), at the margin of the prespecified success criterion (lower bound of credible interval exceeding 0.8) (Table 1). The different-section ICCs for the unweighted global scores and hot-spot scores were 0.86 (95% CI = 0.793–0.92) and 0.83 (95% CI = 0.74–0.90), respectively, and

therefore both these methods had ICC credible intervals that extended below the success criterion at the lower 95% limit. The corresponding same-section ICC estimates for the weighted global, unweighted global and hot-spot scores were virtually identical 0.87 (95% CI = 0.799–0.92), 0.86 (95% CI = 0.79–0.92) and 0.83 (95% CI = 0.74–0.90) respectively, supporting that differences between serial sections were minimal. Figure 1 displays the side-by-side box-plots of Ki67 scores among pathologists (hereafter referred to as ‘observers’) by group. Summary statistics for the Ki67 scores among the 23 observers are given in Supporting information, Tables S2–S4.

The median number of nuclei counted per slide (across all observers and cases) was 400 and 500 for the global and hot-spot methods, respectively. The corresponding minimum number of nuclei counted was 300 and 138. Eighteen per cent of the hot-spot scores were based on < 500 nuclei counts. Among these 126 hot-spot scores, the median number of nuclei counted was 375.

In a context where pre-analytical and staining factors are held constant, variance component analyses show that, regardless of scoring method, biological variation among different patients was the largest component of the total variation on these centrally stained slides, indicating that the Ki67 score is reflecting inherent properties of the tumour (Figure 2, Supporting information, Table S5).

INTEROBSERVER VARIATION OF KI67 SCORING

Figure 3 displays the variation in scores across observers for cases in slide set 1 as spaghetti plots. The corresponding plots for slide sets 2–4 are displayed in Supporting information, Figure S4. Figure 4 presents the scores in a heat-map format with the columns (observers) ordered (within each slide set) by the median scores across cases and the rows (cases) sorted by the median scores across observers.

Overall, it can be seen that most observers show good parallelism in the increasing Ki67 scores throughout the plots. In other words, observers measuring higher or lower than others tended to do so relatively consistently.

CATEGORICAL CONCORDANCE OF KI67 SCORING

Regarding concordance on a categorical level (< 10, 10–20 and > 20%), the relationship between concordance and continuous score is shown in Supporting information, Figure S5. It shows excellent to perfect concordance on cases with scores that are either much lower or higher than the intermediate range (10–20%).

Based on visual inspection of captured images, locations of the hot-spot selections tended to cluster in the same region among observers within each of the excision whole-section slides (Figure 5 shows some examples; virtual slide images of all slides used in this study and the corresponding selected fields and scores can be viewed at <http://www.gpec.ubc.ca/papers/ki67p3b>).

The median scoring time (field selection and nuclear counting) was 9 (interquartile range: 7–11) and 6 (interquartile range = 4–8) minutes for global and hot-spot methods, respectively.

Discussion

The IKWG has demonstrated that it is possible, when controlling stringently for variability due to pre-analytical and analytical aspects of the Ki67 immunohistochemistry assay,⁹ and given a set of clearly defined training exercise and scoring instructions, for pathologists to achieve high interobserver concordance in Ki67 scoring on core-cut biopsies and now on excision whole

sections using a conventional light microscope and manual field selection, with no additional aid such as a counting grid.

Due to the limited sample size, we were unable to assess whether any specific method (weighted global, unweighted global or hot-spot) is significantly more reproducible than others. However, the observed ICCs for global score (weighted = 0.87; unweighted = 0.86) are relatively higher compared to hot-spot score (0.83), suggesting that a sufficiently powered study might be able to show more convincingly whether global scores are more reproducible. This result is consistent with findings on core biopsies.³⁰

Can this level of concordance be clinically adequate? The POETIC¹¹ study assessed Ki67 (cut-point at 10%) as a prognostic marker. Applying this cut-point to the data in our current study, 17 (of 30) cases have, at most, one discordance in weighted global score (Figure 4A). There are cases with major discrepancies: TB036, on the same physical slide (set 2), received a weighted global score of 4 and of 21% from observers A and L, respectively. However, it is apparent (Figure 4) that cases far away from the intermediate range (10–20%) tend to have good agreement. Considering that cases in our current study are a random sampling of the general ER⁺ breast cancer population, one could expect that approximately half of these cases would fall away from the intermediate range, and hence Ki67 may provide clinically adequate information, provided that the staining and pre-analytical factors do not add too much variability.

Are the proposed scoring methods practical? The median scoring time is 6–9 min, depending on the method used. However, an adaptive scoring protocol can be used to reduce scoring time if the purpose is to assess whether Ki67 is above or below a specific cut-point. For example, considering the global scoring method, where the maximum nuclei count is prespecified (i.e. 400), to determine whether a case has unweighted global score $\geq 10\%$ the pathologist can stop

counting if the first field they scored is $\geq 40\%$. For cases with a very low Ki67 score, one would probably still need to count all 400 nuclei.

The proposed scoring protocols do not make any recommendation concerning the required minimum tumour nuclei count. This is a limitation of this study and, in practice, it will be up to the discretion of the scoring pathologist to assess if too few tumour nuclei are available for an adequate Ki67 assessment. This will depend on the percentage of positive cells scored in the cells available and the clinical context for the measurement.

An external quality assessment programme (e.g. NordiQC³⁴), involving comparison of laboratory scores with reference scores in periodic assessment challenges, will probably improve interobserver reproducibility further. Recent studies suggest that an even higher level of concordance can be achieved with automated image analysis.^{35–38} The IKWG is actively conducting studies in this area to assess how artificial intelligence may help to standardise Ki67 assessment.^{35,38} Also, concordance between Ki67 scores on core biopsies and excision specimens is currently being investigated.

In conclusion, this study demonstrates that an adequately high level of interobserver concordance can be achieved by visual assessment of Ki67 using practical scoring methods, although some cases with large discrepancies remain. A two-tier assessment approach may be worthy of further study as a means to reduce scoring burden and further address challenging cases: if the Ki67 value from the initial scoring falls on a grey zone (e.g. cut-point $\pm 5\%$), scoring by a second pathologist or alternative test could be pursued. Pre-analytical and analytical aspects of the immunohistochemistry assay, areas that still need standardisation before the clinical utility of this marker can be proved, will probably add more variability. A clinical validation study employing

analytically reproducible methodology would also need to be completed in appropriate cohorts of cases to determine whether Ki67 can be recommended for patient care decisions.

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Conflicts of interest

S.S.D. has participated in Scientific Advisory Boards/ Speaker for Genomic Health Inc., Dako/Agilent, Roche Diagnostics, Targos GmbH, Athenax, Konica-Minolta and received compensation. S.S.D. has received research funding or in-kind support from Dako/Agilent, and has intellectual property right/ownership interests with IU. He is also associated with two startup companies (SYSGenomics and YeSSGenomics). J.M.S.B has consulted for BioNTech GmbH, Biotheranostics Inc, RNA Diagnostics, and received compensation. He has participated in Scientific Advisory Boards for Biotheranostics and RNA Diagnostics and received compensation and has received research funding or in-kind support from Nanostring, Biotheranostics Inc, BioNTech GmbH. He has intellectual property right/ownership interests with OICR/FACIT. S.B.

has participated in educational talks/covered scientific conferences by Roche and Novartis. M.D. is on the Oncology Advisory Board for Radius and has provided *ad-hoc* advice to Orion and Gtx. He has received lecture fees from Myriad and Roche and institutional research grants from Radius, AstraZeneca and Puma. He receives income from the Institute of Cancer Research Rewards for Inventors Scheme (abiraterone). A.E. has participated in educational talks organised by Roche but without economical compensation. S.F. participated in a scientific advisory board for Genomic Health and has received monetary compensation (not for salary). D.F.H. reports research support from Menarini Silicon Biosystems (MSB), Merrimack, Eli Lilly, Puma Biotechnology, Pfizer, AstraZeneca. He is the named inventor of patent US 8,790,878 B2. D.H.F. which is licensed to MSB and from whom he receives royalties. He holds stock options from OncImmune LLC and InBiomotion, and he serves as a paid advisor for Cepheid, Freenome, CellWorks, Agendia and CVS Caremark. A.-V.L. has received research funding from Nanostring Technology (not for personal salary), participated in advisory board for Roche A/S and Novartis (for purely scientific reasons; honoraria declined) and received travel expenses for congress attendance from Astra Zeneca and Roche A/S (past 2 years). T.O.N. has consulted for Nanostring and received compensation. He has intellectual property rights/ownership interests from Bioclassifier LLC. C.K.O. has consulted for Astra Zeneca, Genentech and NanoString and received compensation. F.M.P.-L. has participated in Scientific Advisory Boards for Nanostring, Myriad, Genomic Health, Agendia, AstraZeneca, Roche, Sanofi, Novartis, Pfizer, BionTech and received compensation. He has received research funding or in-kind support from Nanostring, AstraZeneca, Roche, BionTech. B.V.d.V. has consulted for Philips and received compensation. All other authors declare no conflict of interest.

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Figure 1. Ki67 scores of all 23 observers (by slide set). Observers are ordered (within each group) by the median scores. The bottom/top of the box in each box plot represent the first (Q1)/third (Q3) quartiles, the bold line inside the box represents the median and the two bars outside the box represent the lowest/highest datum still within $1.5 \times$ the interquartile range (Q3–Q1). Outliers are represented with empty circles.

Figure 2. Variance component analysis. Variation due to different components are presented in a bar plot to show the relative magnitude of differences between them. Numerical values of the variance components estimates and the corresponding credible intervals are shown in Supporting information, Table S5.

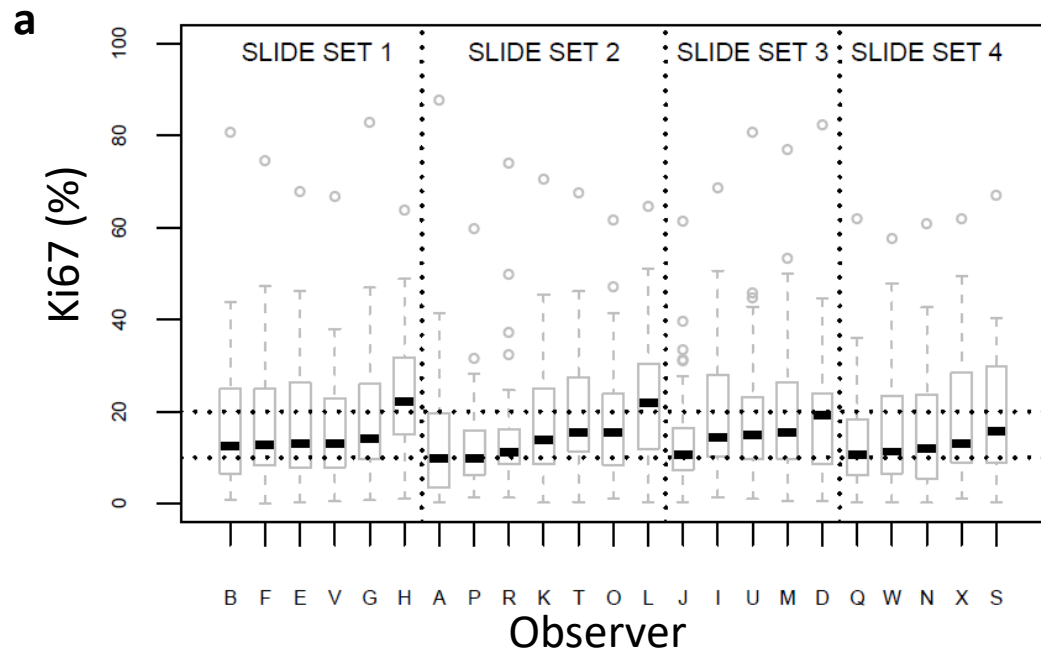
Figure 3. Variability in Ki67 scores (slide set 1 only). Each line represents Ki67 scores from one observer. Shaded region indicates Ki67 scores between 10 and 20%. Scores on slide sets 2–4 are shown in Supporting information, Figure S4.

Figure 4. Heat-map of Ki67 scores (**A**, weighted global; **B**, unweighted global; **C**, hot-spot). Rows represent cases and columns represent observers. Green colour indicates that the score is < 10%, yellow 10–20% and red > 20%. Cases are ordered by the median scores (across observers), which are shown in parentheses beside the specimen number. Observers are ordered (within each group) by the median scores (across cases). The three colon-separated numbers to the right of the heat-map represent the number of observers giving scores falling into different ranges: < 10% (left), 10–20% (middle) and > 20% (right). For example, ‘15:6:1’ indicates that 15 observers gave a score of < 10%, six observers between 10 and 20% and one observer > 20%.

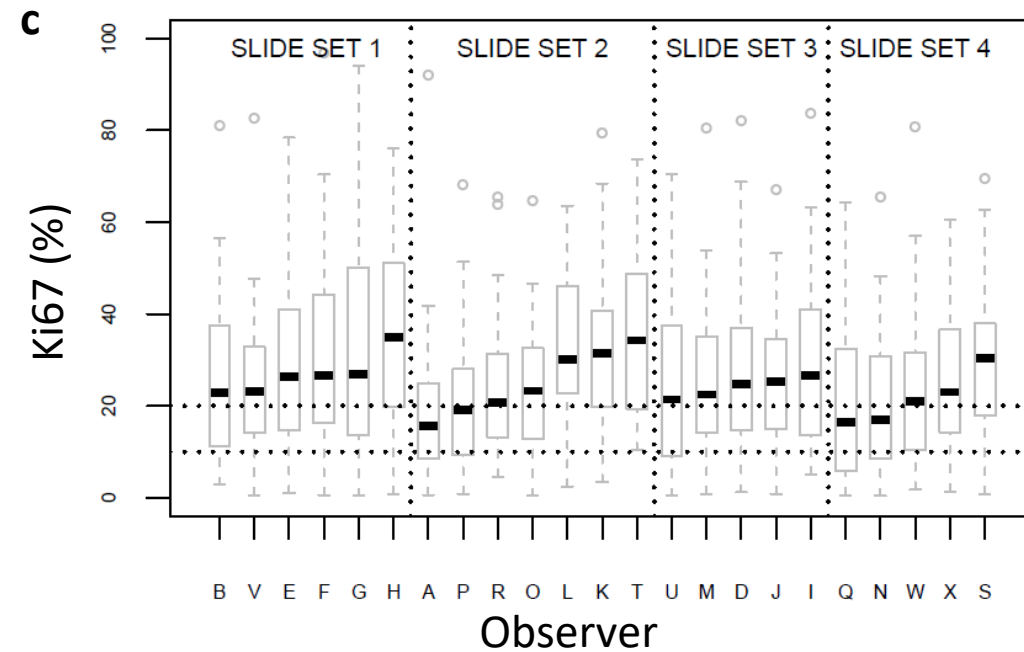
Figure 5. Hot-spot field selection by different observers on the same excision whole section slide. **A**, Selections (indicated by red circles) on some example excision whole section slides. **B**, An example of a single excision whole section slide (median score: 18%) with zoomed-in fields. Each observer was asked to circle the area considered to be the hot-spot (**B-i**). Most observers honed in on the same general area of the slide, although individual selected scoring fields do not always

478 overlap. **B-iii** and **B-iv** represent segments of the same area chosen by two different observers to
479 read Ki67. Figure **B-v** represents the ‘outlier’ field selected by only one observer as the hot-spot.

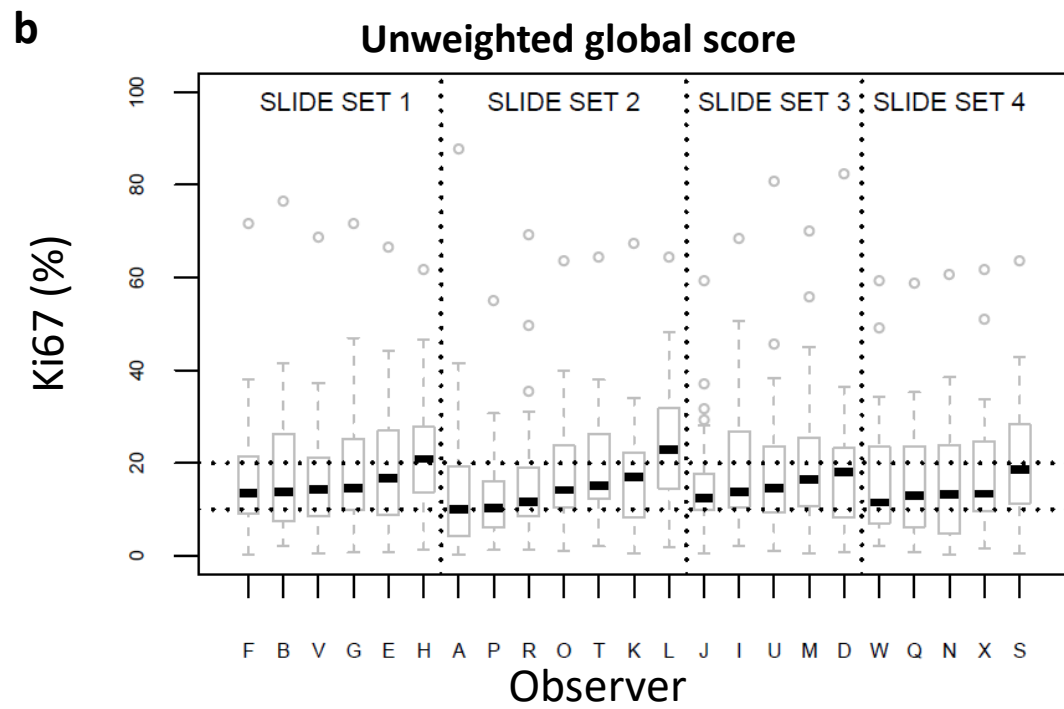
Weighted global score



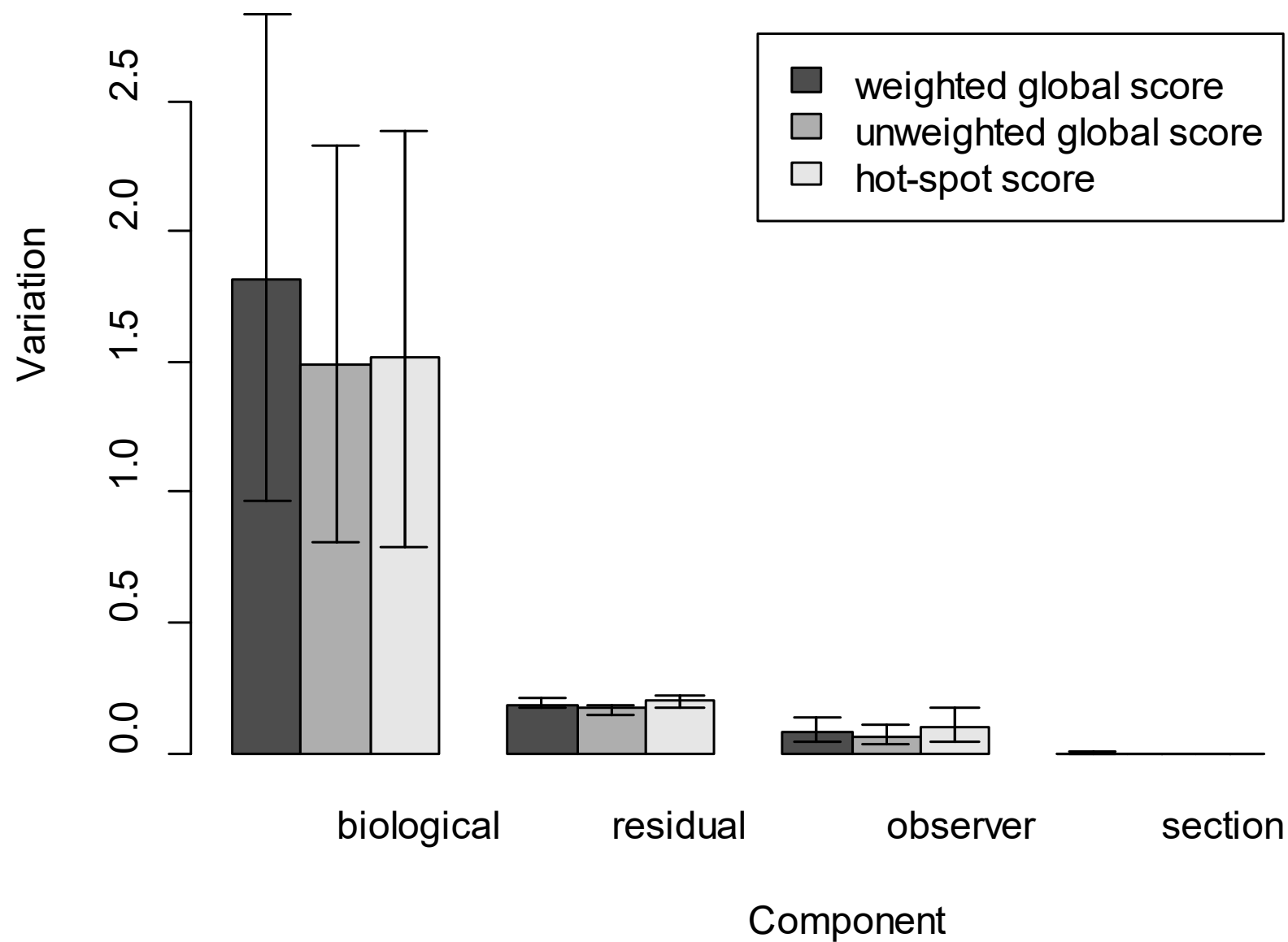
Hot-spot score

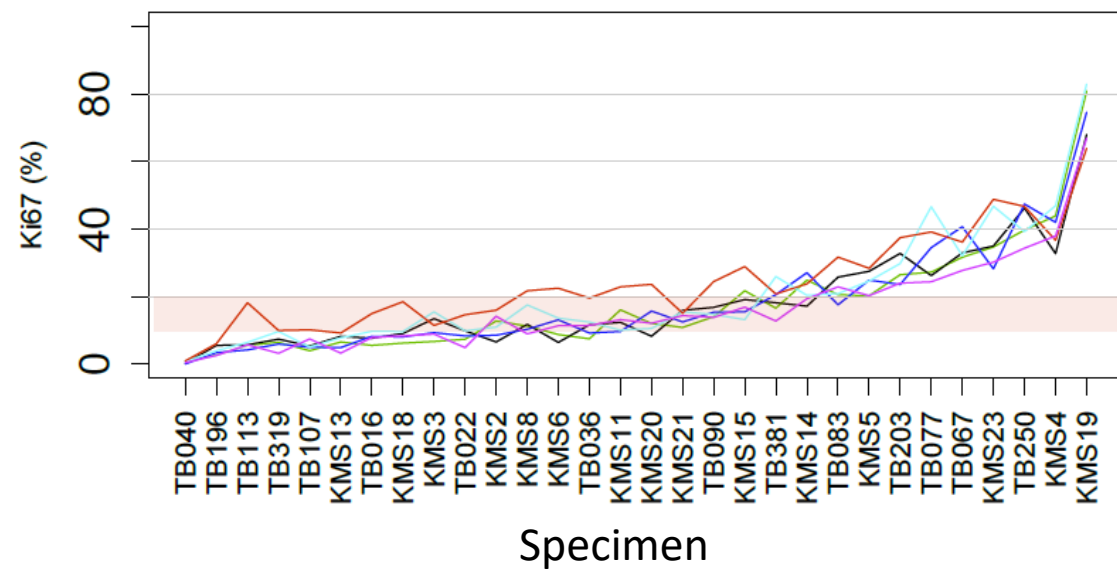
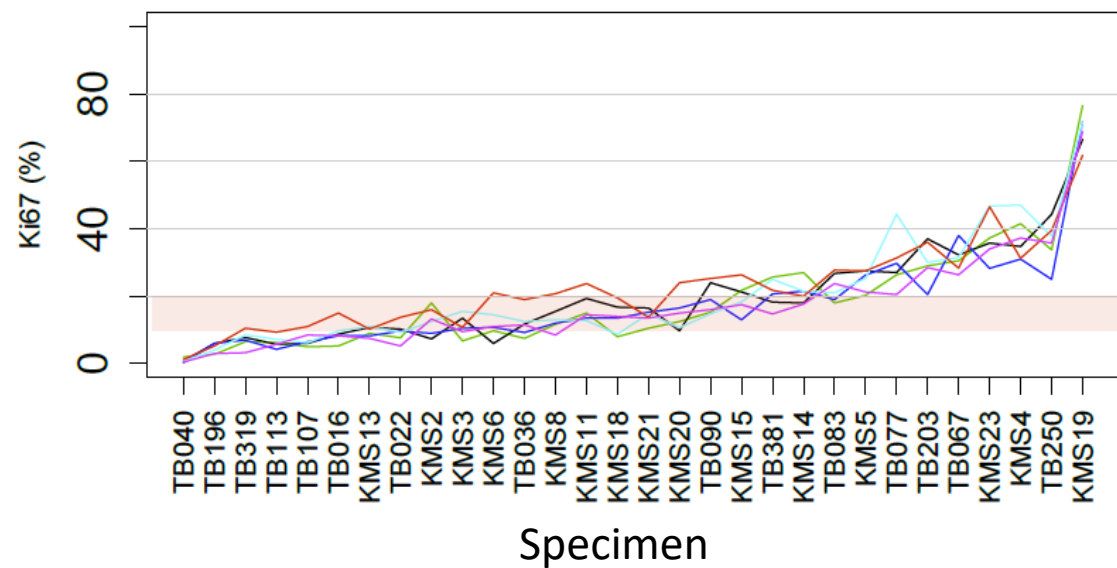
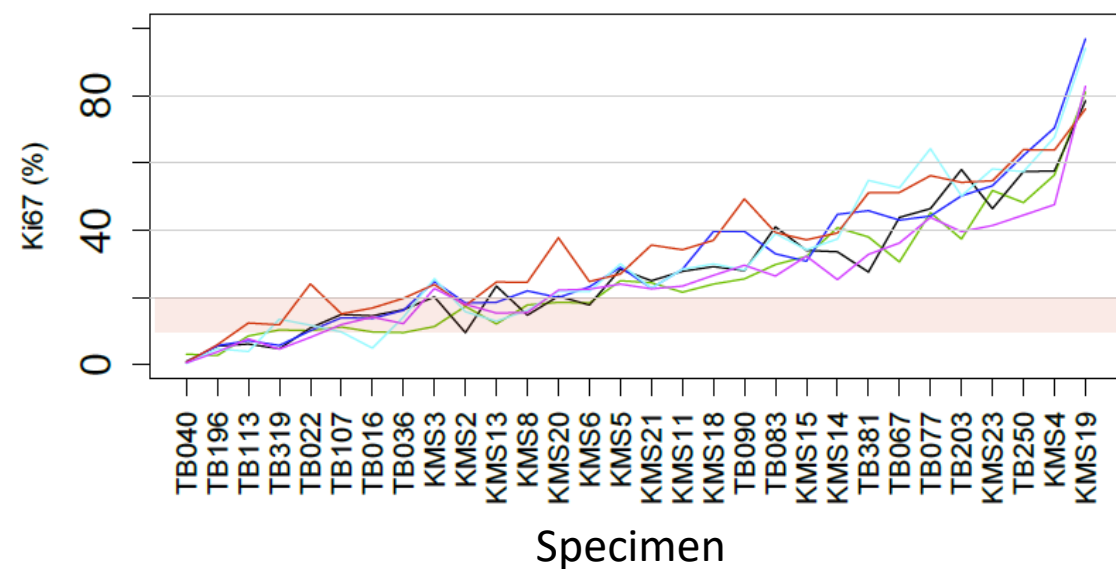


Unweighted global score



Variance component analysis



a**Weighted global score****b****Unweighted global score****c****Hot-spot score**

a. Weighted global score

	(median score)	SLIDE SET 1						SLIDE SET 2						SLIDE SET 3					SLIDE SET 4						
		B	F	E	V	G	H	A	P	R	K	T	O	L	J	I	U	M	D	Q	W	N	X		S
Specimen	TB040 (0)	1	0	0	0	1	1	0	1	1	0	0	1	0	0	1	1	0	1	0	0	0	1	0	23:0:0
	TB196 (3)	3	3	6	3	4	6	2	2	5	2	3	4	3	1	4	2	4	2	4	3	4	2	2	23:0:0
	TB113 (6)	6	4	6	6	6	18	4	6	5	6	8	8	9	3	6	6	7	5	4	5	3	6	7	22:1:0
	TB319 (6)	6	6	7	3	10	10	2	5	5	5	6	8	7	4	4	6	6	9	6	7	6	8	4	21:2:0
	TB107 (6)	4	5	5	8	5	10	3	3	9	6	8	6	8	3	8	6	8	7	5	5	4	7	9	22:1:0
	KMS13 (7)	7	5	8	3	8	9	2	2	8	4	11	12	9	4	10	17	11	4	8	7	3	4	6	18:5:0
	TB016 (8)	6	8	8	8	10	15	5	7	8	9	11	13	21	8	13	10	10	10	6	8	7	12	7	13:9:1
	KMS18 (9)	6	8	9	8	10	18	3	12	9	11	16	6	19	6	8	9	10	16	10	5	2	2	5	14:9:0
	KMS3 (9)	7	9	14	9	16	12	7	7	9	7	9	8	12	9	16	12	15	12	6	11	9	11	9	13:10:0
	TB022 (9)	7	8	10	5	10	15	3	10	6	13	15	5	5	8	10	7	9	7	10	6	11	11	14	12:11:0
	KMS2 (10)	13	9	7	14	11	16	5	9	8	14	13	11	20	9	10	10	9	11	6	9	4	11	10	10:13:0
	KMS8 (10)	11	10	12	9	18	22	9	10	10	18	15	8	22	10	11	17	10	14	8	7	9	9	10	7:14:2
	KMS6 (12)	9	13	6	11	14	22	12	11	11	9	16	19	20	10	9	10	15	24	12	11	8	18	15	5:16:2
	TB036 (12)	8	9	12	12	12	20	4	5	13	11	14	13	21	25	15	14	12	12	9	9	8	13	10	7:14:2
	KMS11 (12)	16	10	12	13	10	23	12	14	10	11	14	10	22	7	14	19	16	7	10	12	11	11	14	2:19:2
	KMS20 (14)	12	16	8	12	11	24	10	9	12	13	16	18	24	11	12	16	15	19	36	13	19	14	17	2:18:3
	KMS21 (15)	11	13	16	14	16	15	11	6	11	21	30	19	24	10	15	15	20	22	11	10	15	13	17	1:18:4
	TB090 (16)	14	15	17	14	15	24	20	16	11	17	15	11	16	11	21	10	22	21	14	14	20	20	22	0:18:5
	KMS15 (17)	22	16	19	17	13	29	8	7	12	19	21	19	21	15	17	14	23	19	16	12	13	16	24	2:15:6
	TB381 (18)	17	21	18	13	26	21	16	10	15	14	20	20	29	11	14	20	28	21	14	10	20	11	21	0:16:7
	KMS14 (19)	25	27	17	19	20	24	10	12	15	15	14	28	28	16	24	15	26	20	15	24	19	18	25	0:14:9
	TB083 (20)	21	18	26	23	21	32	25	10	19	19	24	19	29	15	23	17	18	20	9	16	24	28	17	1:11:11
	KMS5 (25)	20	25	28	20	25	28	18	14	13	25	28	18	31	11	28	28	25	21	18	22	18	29	30	0:9:14
	TB203 (26)	26	24	33	24	30	37	18	19	16	26	25	26	45	31	34	37	33	24	23	34	43	18	32	0:4:19
	TB077 (30)	27	34	26	24	47	39	27	18	19	32	33	28	30	15	31	36	23	45	18	34	30	31	30	0:4:19
	TB067 (32)	32	41	33	28	32	36	24	25	25	32	46	24	45	28	31	23	38	33	29	30	29	34	33	0:0:23
	KMS23 (34)	35	28	35	30	47	49	24	25	32	30	38	35	46	34	39	43	45	34	30	34	34	34	40	0:0:23
	TB250 (40)	40	47	46	34	39	47	42	32	37	39	44	41	51	40	50	46	50	35	27	32	40	31	40	0:0:23
	KMS4 (42)	44	42	33	38	47	37	29	28	50	45	28	47	50	31	51	45	53	39	25	48	27	49	33	0:0:23
	KMS19 (68)	81	75	68	67	83	64	88	60	74	70	68	62	64	61	68	81	77	82	62	58	61	62	67	0:0:23

b. Unweighted global score

Specimen	(median score)	SLIDE SET 1						SLIDE SET 2						SLIDE SET 3					SLIDE SET 4						
		F	B	V	G	E	H	A	P	R	O	T	K	L	J	I	U	M	D	W	Q	N	X		S
TB040 (1)		0	2	0	1	1	1	0	1	1	1	2	0	2	0	2	1	0	1	2	1	0	2	0	23:0:0
TB196 (4)		6	3	3	4	6	5	2	2	5	5	4	2	2	4	4	2	4	2	3	4	4	5	4	23:0:0
TB319 (6)		7	6	3	8	8	10	2	4	5	9	7	5	8	4	4	6	7	8	6	6	5	8	4	22:1:0
TB113 (6)		4	6	6	7	6	9	4	7	5	8	8	6	8	4	6	6	7	5	5	4	3	6	8	23:0:0
TB107 (6)		6	5	8	6	6	11	3	3	10	6	8	8	8	2	8	6	10	7	5	5	4	7	10	19:4:0
TB016 (8)		8	5	8	10	9	15	6	7	8	13	11	8	21	7	13	10	10	9	8	6	6	12	8	14:8:1
KMS13 (8)		8	9	8	11	11	10	4	5	8	13	15	6	14	6	12	15	12	4	8	8	5	4	11	13:10:0
TB022 (10)		10	8	5	9	10	14	4	10	6	4	14	12	8	10	10	7	11	8	6	10	11	11	14	10:13:0
KMS2 (10)		9	18	13	12	7	16	5	10	8	11	13	15	21	10	10	9	10	12	10	6	5	11	11	7:15:1
KMS3 (10)		10	7	10	16	14	11	5	6	9	8	12	8	14	10	15	12	16	11	11	6	9	11	10	8:15:0
KMS6 (11)		11	10	11	14	6	21	14	9	10	19	17	10	18	11	9	10	16	21	10	12	7	17	18	4:17:2
TB036 (12)		9	8	12	12	12	19	4	5	12	13	14	10	23	29	15	14	12	12	9	9	8	13	12	7:14:2
KMS8 (12)		12	12	8	13	16	21	9	10	11	10	12	18	23	12	11	14	12	14	7	8	9	10	14	5:16:2
KMS11 (12)		14	15	14	13	19	24	12	11	12	10	13	12	27	11	12	20	13	8	12	12	10	11	15	1:20:2
KMS18 (14)		14	8	14	9	17	20	4	16	15	11	16	15	20	12	12	8	16	16	6	24	4	2	12	7:15:1
KMS21 (15)		15	10	14	15	16	14	11	6	11	18	26	19	27	12	14	15	19	21	11	13	15	13	20	1:19:3
KMS20 (15)		16	12	15	11	10	24	12	10	12	17	13	16	28	14	11	16	16	18	13	35	18	14	19	0:20:3
TB090 (18)		19	15	16	15	24	25	18	16	10	12	15	18	17	16	21	12	20	23	16	18	19	20	28	0:18:5
KMS15 (18)		13	22	18	18	21	26	8	10	19	19	25	18	22	16	16	14	22	19	15	16	16	17	26	1:15:7
TB381 (18)		21	26	15	25	18	22	19	10	17	15	23	18	32	14	13	18	30	22	12	16	20	16	22	0:14:9
KMS14 (20)		22	27	18	22	18	20	9	14	15	26	15	18	30	15	24	15	26	20	24	16	23	20	28	1:12:10
TB083 (21)		19	18	24	21	27	28	22	14	20	16	22	22	32	16	20	14	20	21	18	10	24	25	23	0:11:12
KMS5 (22)		26	20	21	25	28	28	15	14	13	16	24	22	31	11	27	28	24	18	22	20	18	29	29	0:9:14
TB077 (27)		30	26	20	44	27	31	19	22	19	28	34	24	32	18	27	36	22	28	32	24	30	28	23	0:4:19
TB203 (28)		20	29	28	30	37	36	23	20	20	24	27	24	40	27	34	33	33	25	34	24	34	22	31	0:3:20
TB067 (30)		38	30	26	32	32	28	24	22	25	25	33	30	42	26	30	24	36	30	30	31	30	34	29	0:0:23
KMS23 (34)		28	37	34	47	36	46	24	25	31	32	38	30	46	32	37	38	44	35	32	27	33	34	43	0:0:23
KMS4 (36)		31	42	37	47	35	31	32	27	50	40	33	34	44	28	48	38	56	36	49	28	24	51	36	0:0:23
TB250 (37)		25	34	36	38	44	40	42	31	36	39	36	34	48	37	50	46	45	36	30	22	38	33	37	0:0:23
KMS19 (66)		72	76	69	72	66	62	88	55	69	64	64	67	64	59	68	81	70	82	59	59	61	62	64	0:0:23

c. Hot-spot score

Specimen	(median score)	SLIDE SET 1						SLIDE SET 2							SLIDE SET 3					SLIDE SET 4					
		B	V	E	F	G	H	A	P	R	O	L	K	T	U	M	D	J	I	Q	N	W	X	S	
TB040 (1)		3	1	1	1	0	1	1	1	6	0	2	3	15	0	1	1	1	6	1	0	2	1	1	22:1:0
TB196 (6)		3	4	6	6	5	6	3	4	7	3	7	6	14	0	7	7	8	11	1	5	7	8	10	20:3:0
TB113 (8)		9	8	6	7	4	12	9	5	9	7	7	16	10	9	12	12	8	5	5	4	6	14	16	16:7:0
TB319 (9)		10	5	5	6	14	12	5	3	14	7	12	9	14	7	11	15	8	10	4	4	6	10	15	12:11:0
TB022 (10)		10	8	11	10	12	24	8	9	15	8	22	20	20	10	15	10	14	6	8	8	9	14	16	8:13:2
TB107 (12)		11	12	15	14	10	15	5	7	13	7	14	11	19	9	13	11	15	11	5	6	12	14	18	6:17:0
TB016 (14)		10	14	15	14	5	17	11	9	12	13	24	15	15	10	9	19	20	17	7	11	10	17	14	4:18:1
TB036 (14)		10	12	16	16	14	20	7	8	15	13	24	19	14	7	14	19	14	13	11	9	11	18	19	4:18:1
KMS3 (14)		11	23	20	25	26	24	8	12	5	16	29	24	14	9	20	12	20	14	4	9	9	11	22	6:10:7
KMS2 (16)		17	18	10	18	16	18	9	13	13	11	23	27	24	15	13	17	17	16	9	8	8	15	20	4:16:3
KMS13 (18)		12	15	23	19	13	25	12	18	21	18	31	26	31	18	16	6	24	21	2	14	13	17	15	2:13:8
KMS8 (18)		18	16	15	22	16	25	17	15	21	18	20	27	26	18	23	21	13	20	6	12	10	14	19	1:15:7
KMS20 (22)		19	22	20	20	22	38	17	20	20	23	35	27	29	22	22	27	25	29	21	17	22	23	28	0:7:16
KMS6 (22)		18	22	18	23	22	25	13	16	12	27	25	25	32	22	26	26	26	14	8	15	18	18	30	1:9:13
KMS5 (24)		25	24	28	29	30	27	23	15	14	23	33	35	37	28	21	24	22	34	24	17	20	23	33	0:4:19
KMS21 (24)		24	23	25	23	23	36	14	23	19	23	27	30	31	16	26	23	31	27	14	16	26	25	30	0:5:18
KMS11 (26)		22	23	28	28	28	34	14	12	29	17	35	33	39	29	21	26	25	26	15	21	30	25	33	0:4:19
KMS18 (28)		24	27	29	40	30	37	23	27	29	28	28	40	39	7	22	35	35	38	18	21	20	19	33	1:3:19
TB090 (30)		26	30	28	40	28	49	25	28	30	27	32	39	50	30	26	32	26	36	31	22	28	30	31	0:0:23
TB083 (31)		30	26	41	33	39	39	22	28	31	33	47	41	46	21	25	33	28	37	27	31	25	31	32	0:0:23
KMS15 (32)		32	32	34	31	34	37	13	26	30	27	25	39	39	30	26	32	32	27	27	18	32	37	37	0:2:21
KMS14 (34)		41	25	34	45	37	39	19	22	21	30	34	38	43	30	33	21	35	42	23	21	26	38	38	0:1:22
TB381 (35)		38	33	28	46	55	51	27	27	31	33	46	41	46	38	35	39	34	39	32	28	26	30	37	0:0:23
TB067 (43)		31	36	44	43	53	51	31	37	49	43	40	46	52	43	49	37	39	54	39	32	47	36	46	0:0:23
TB077 (45)		45	44	46	44	64	56	25	36	43	31	50	50	49	44	52	46	34	41	43	33	52	49	48	0:0:23
TB203 (47)		37	40	58	50	50	54	39	42	42	41	47	50	50	45	48	40	48	51	44	47	48	46	52	0:0:23
KMS23 (48)		52	41	46	53	58	55	42	34	42	36	52	48	55	55	52	41	51	47	46	43	43	48	57	0:0:23
TB250 (53)		48	44	57	62	57	64	32	43	48	47	51	60	54	56	54	69	53	57	40	48	49	53	49	0:0:23
KMS4 (56)		56	48	58	70	68	64	38	51	64	45	58	68	54	48	53	53	51	63	55	39	57	61	63	0:0:23
KMS19 (76)		81	83	78	97	94	76	92	68	66	65	64	79	74	71	81	82	67	84	64	66	81	56	70	0:0:23

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